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Cloning of Mid-G_1 Serum Response Genes and Identification of a Subset Regulated by Conditional myc Expression

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The emergence of cells from a quiescent G_0 arrested state into the cell cycle is a multistep process that begins with the immediate early response to mitogens and extends into a specialized G_1 phase. Many immediate early serum response genes including c-fos, c-myc, and c-jun are transcriptional regulators. To understand their roles in regulating cell cycle entry and progression, the identities of their regulatory targets must be determined. In this work we have cloned cDNA copies of messenger RNAs that are either up- or down-regulated at a mid-G_1 point in the serum response (midserum-response [mid-SR]). The mid-SR panel is expected to include both direct and indirect targets of immediate early regulators. This expectation was confirmed by the identification of several transcriptional targets of conditional c-myc activity. In terms of cellular function, the mid-SR class is also expected to include execution genes needed for progression through G_1 and into S-phase. DNA sequence data showed that the mid-SR panel included several genes already known to be involved in cell cycle progression or growth transformation, suggesting that previously unknown cDNAs in the same group are good candidates for other G_1 execution functions. In functional assays of $G_0 \rightarrow$ S-phase progression, c-myc expression can bypass the requirement for serum mitogens and drive a large fraction of G_0 arrested cells through G_1 into S-phase. However, beyond this general similarity, little is known about the relation of a serum-driven progression to a myc-driven progression. Using the mid-SR collection as molecular reporters, we found that the myc driven G_1 differs qualitatively from the serum driven case. Instead of simply activating a subset of serum response genes, as might be expected, myc regulated some genes inversely relative to serum stimulation. This suggests that a myc driven progression from G_0 may have novel properties with implications for its action in oncogenesis.

INTRODUCTION

When exponentially growing NIH or Balb/c 3T3 cells are deprived of serum growth factors, cell cycle progression halts after mitosis and the cells enter a proliferation arrested state termed G_0. Restoration of mitogens activates signaling cascades that stimulate emergence from G_0 growth arrest and support further cell cycle progression (for review, see Pardee, 1989). The G_1 phase that follows is specialized and temporally protracted compared with the G_1 of cycling cells. The $G_0 \rightarrow$S-phase progression is of interest because for many vertebrate cell types it is the major decision point regulating cell proliferation and execution of cell differentiation programs.

At the molecular level, a good deal is now known about the earliest events at the G_0/G_1 transition, but less is known about subsequent events in the specialized G_1 that follows. The initial response of G_0 arrested cells to serum growth cues is characterized by rapid, robust, protein-synthesis-independent up-regulation of transcription from a specific set of genes (Lau and Nathans,

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1987; Almendral *et al.*, 1988; Mohn *et al.*, 1991). Prominent among these immediate early response genes are known or suspected transcription factors of the AP-1, myc, nuclear receptor, and zinc finger families (for review, see Bravo, 1990). Their dramatic synthesis at the G_0/G_1 transition suggests that they are members of a regulatory heirarchy that initiates progression through G_1. From a cell biological perspective, it is now important to identify genes that are up- or down-regulated during mid-G_1 of the serum response (midserum-response [mid-SR]) and define their individual and collective roles in execution of cell proliferation. From a molecular perspective, these mid-SR genes are likely to include physiological targets of the immediate early transcription regulators and as such serve as natural reporters for their function. A primary goal of this work was to expand the repertoire of cloned mid-SR genes.

Ectopic expression of c-myc in G_0 arrested cells can drive them through G_1 and into S-phase (Cavalieri and Goldfarb, 1987; Eilers *et al.*, 1991). Myc is a component of a sequence specific DNA binding transcription factor. In vitro studies have shown that c-myc homodimerizes poorly and as a result binds its preferred site, CACGTG, inefficiently (Blackwell *et al.*, 1990). But in collaboration with its pairing partner, Max/myn (Blackwood and Eisenman, 1990; Prendergast *et al.*, 1991) it forms heteromers that bind DNA very efficiently. This protein: protein association is likely to be physiologically relevant because it is also observed in vivo (Wenzel *et al.*, 1991; Blackwood *et al.*, 1992). In serum stimulated NIH or Balb/c 3T3 cells, the temporal pattern of immediate early c-myc expression and delayed early Max/myn expression (Prendergast *et al.*, 1991) suggests that the activity of a putative c-myc:Max/myn heteromeric transcription factor will be stimulated by serum and will peak during the extended G_1 that follows. This leads to the hypothesis that myc, in association with Max/myn or some similar partner, is responsible for regulating an identifiable subset of the mid-SR genes. Furthermore, the myc responsive gene class is sufficient to drive progression from G_0 to S. Therefore, a second goal in this work was to identify mid-SR genes that are subject to myc regulation.

In this work, we prepared cDNA libraries from growth arrested and mid-G_1 serum stimulated 3T3 cells, differentially screened those libraries with corresponding cDNA probes, and obtained a panel of cDNA clones subject to either up- or down-regulation with mid-SR kinetics. These mid-SR genes were subsequently screened to identify a subset that are regulated in response to conditional c-myc expression. Several were identified and shown to be transcriptionally regulated in response to myc. The mid-SR panel was also used to evaluate the similarity of a myc-driven G_1 to a serum driven G_1. Several genes responded similarly to serum and conditional c-myc activity and are therefore candidates for common execution functions for progression. Others, however, responded inversely to the two stimuli, suggesting that the G_1 progression elicited by myc differs qualitatively from the serum response.

MATERIALS AND METHODS

Recombinant Plasmids Used in Stable Transfections

Plasmid myc23c encodes the mouse c-myc proto-oncogene and was used to stably transfect NIH 3T3 cells. The plasmid was assembled in several steps in pT7T3-18 (Bethesda Research Laboratories, Gaithersburg, MD). From 5′ to 3′, it consists of a 756 bp *Taq* I-*Sac* I fragment from the MTV LTR bearing a glucocorticoid regulated enhancer (Payvar *et al.*, 1983), a 218 bp *Sac* I-*Bgl* II fragment containing the mouse metallothionein I (Mt-I) promoter (Mueller *et al.*, 1988), a 550 bp *Xba* I-*Sac* II fragment from the first intron and second exon of the mouse c-myc genomic clone lambda myc 5 (Hood and Barth, unpublished observations), a 922 bp *Sac* II-*Nsi* I fragment containing the remainder of the c-myc coding sequence derived from the c-myc cDNA myc 29 (Mueller, Kim, and Wold, unpublished observations), and a 302 bp *Hgi* AI-*Hind*III fragment containing the poly-A addition signal from the mouse Mt-I gene (Mueller *et al.*, 1988). The *Taq* I site at the 5′ end of the enhancer fragment was filled-in with Klenow polymerase and ligated to a filled-in *Eco*RI site within the polylinker, regenerating the *Eco*RI site. The *Bgl* II site at the 3′ end of the Mt-I promoter was ligated into the *Bam*HI site of the polylinker, destroying both sites; similarly, the *Nsi* I-*Hgi* AI fusion between the 3′ end of the myc cDNA fragment and the 5′ end of the Mt-I poly-A addition fragment destroyed both sites. Plasmid pNeo 3, an HSV-tk promoted neo construct (Bond and Wold, 1987), was used as a G418 selectable marker in the preparation of stably transfected cell lines.

Stable Transfections

At the outset of this work, NIH 3T3 cells and c-myc transfected derivatives were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 U of penicillin G per milliliter, and 7.5 U of streptomycin per milliliter. Cells were transfected with a calcium phosphate coprecipitate of plasmid and carrier DNAs by a standard procedure (Wigler *et al.*, 1979). Using pNeo 3 as a selectable marker, NIH 3T3 cells co-transfected with pmyc23c were selected for growth in G418 (Gibco, Grand Island, NY). In the first two sets of transfections, relatively fast growing clones were chosen for individual analysis, while remaining clones were pooled. Of more than fifteen individual clones checked, none synthesized more RNA from the myc23c transgene than from their endogenous c-myc gene. In contrast, the pooled cell lines expressed considerably more myc23c RNA. In a third transfection, slow growing clones were deliberately selected. Of 10 clones tested, 4 expressed high levels of the myc23c mRNA. Initial analysis of RNA from these cells using probes sensitive to the 5′ and 3′ ends of the myc23c transcript showed that the predicted transcript was made. Subsequently, several individual myc23c expressing lines were reisolated from the myc23c expressing pools.

Cell Culture

Routine culture of NIH 3T3, c-myc transfected derivatives thereof, and Balb/c 3T3 cells was in DMEM with 15 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethamesulfonic acid (HEPES) (Irvine Scientific, Santa Ana, CA) supplemented with 10% calf serum (Gibco) and 1× glutamine/pen/strep (Irvine Scientific). For experiments comparing Balb/c 3T3 cells with those expressing a c-myc/estrogen receptor fusion protein (MER #6 cells) (Eilers *et al.*, 1989, 1991), both lines were maintained in phenol red free DMEM supplemented with 5% calf serum (Gibco), 4 mM HEPES free acid, and 1× glutamine/pen/strep.

Two growth arrest protocols used were the following: 1) low-density arrest in defined medium and 2) arrest by contact inhibition in medium

containing 5% calf serum. Culture in defined medium was essentially as in Zhan and Goldfarb (1986), with the modifications noted below. Tissue culture dishes (15 cm and 24 well, Nunc) were first coated with polylysine (1 mg/ml in H_2O, Sigma) for 2–3 hours at 37°, rinsed with phosphate buffered saline (PBSA), and coated overnight at room temperature with bovine fibronectin (15 μg/ml in PBSA, Sigma). For cell cycle progression work and RNA preparation, defined medium (DMI-2) consisted of DMEM F-12 HEPES (Flow Laboratories, McLean, VA), 4 μM manganese chloride, 10 μM ethanolamine, 100 nM sodium selenite, 1× glutamine (Irvine Scientific), 5 μg/ml apotransferrin (Sigma), and 500 μg/ml bovine serum albumin-linoleic acid complex (Sigma). Hormonal supplements were 30 ng/ml insulin and 10 pg/ml TGF-β (RD Systems, Minneapolis, MN). We found that low concentrations of TGF-β, which should facilitate assembly of exogenous fibronectin (Allen-Hoffman *et al.*, 1988), improved cell survival through the timecourse of the experiments. We did not add histidine or hydrocortisone.

For defined medium based experiments comparing Balb/c 3T3 cells with MER #6 cells, defined medium (DMI-3) consisted of 1:1 phenol red free DMEM and phenol red free F12 (Gibco), 10 mM HEPES pH 7.2, 4 μM manganese chloride, 10 μM ethanolamine, 100 nM sodium selenite, 1× glutamine/0.1× pen/strep (Irvine Scientific), 5 μg/ml apotransferrin, 500 μg/ml bovine serum albumin-linoleic acid complex, 30 ng/ml insulin, and 10 pg/ml TGF-β.

For density arrest-based experiments comparing Balb/c 3T3 cells with MER #6 cells, cells were brought to growth arrest on fibronectin coated plates in phenol red free DMEM that had been adjusted to 4 mM HEPES with the addition of dry HEPES free acid, 5% calf serum, 1× glutamine/pen/strep, and 1× GMS-X medium supplement (Gibco).

RNA Preparation

Polylysine/fibronectin coated 15-cm plates were seeded at a density of 3×10^6 cells per plate in 25 ml of DMEM plus 10% calf serum. Twelve hours later the cells were washed with PBS and fed with 25 ml of DMI (defined medium). Twelve hours later the cells were refed with 25 ml of DMI. Twenty-four hours later the cells were serum induced with the addition of FBS to 10% final concentration, zinc induced with the addition of various concentrations of zinc sulfate (diluted in DMEM phosphate free HEPES [Irvine]), or mock induced with the addition of an appropriate volume of DMEM phosphate free HEPES.

When RNA was intended for library or probe preparation, cells were harvested in 5 M guanidinium thiocyanate, 50 mM tris(hydroxymethyl)aminomethane (*Tris*) pH 7.5, 12.5 mM EDTA, 2% *N*-lauryl sarcosine, and 1% 2-mercaptoethanol. Lysates were adjusted to 1.5 μg/ml ethidium bromide and an approximate density of 1.6 g/ml with CsTFA (Pharmacia, Piscataway, NJ). Lysates were spun at 41,500 rpm for 30 h in an SW55 rotor, the RNA band was withdrawn, ethanol precipitated twice, and redissolved in 10 mM Tris, 1 mM EDTA, pH = 7.8 (TE) (Mirkes, 1985). RNA intended for ribonuclease (RNase) protections was prepared by a modified guanidinium thiocyanate/organic extraction protocol (Chomczynski and Sacchi, 1987).

cDNA Clone Selection and Management

1) Poly A plus RNAs were prepared from growth arrested and 8 h serum-stimulated Balb/c 3T3 cells. 2) First strand cDNAs were eventually used as probes. Hemimethylated double stranded cDNAs were directionally cloned into the vector λ EXLX+ (Palazzolo *et al.*, 1990). 3) Biotinylated cRNA was prepared by transcribing phage library DNAs en masse with T7 RNA polymerase and a nucleotide mixture containing biotinylated UTP (BRL). Biotinylated cRNA from the growth arrested library was hybridized to serum stimulated cDNA and biotinylated cRNA from the serum stimulated library was hybridized to growth arrested cDNA. Heteroduplexes and unhybridized cRNA were removed with the addition of streptavidin followed by phenol extraction. 4) The resultant subtracted cDNAs were ^{32}P labeled by priming with random nonamers and extending with T7 DNA polymerase. A "backprobe" was generated by similarly labeling a limited collection of serum regulated clones plus clones selected in preceeding rounds of library screening (see step 6). 5) λ libraries were plated at a density of 1000 plaques per 15-cm plate. Three lifts were made from each plate. One lift was probed with the growth arrested subtracted probe, a second lift was probed with the serum stimulated subtracted probe, and a third lift was probed with the backprobe. Plaques differentially hybridized to by the first and second probes and not labeled by the backprobe were picked for further analysis. 6) cDNA inserts were PCR amplified from primary plaques using primers based on flanking vector sequences. The 3′ end primer was ^{32}P labeled, resulting in 3′ end labeled cDNAs. After gel purification, fragments were subjected to partial restriction digest mapping from their 3′ ends with *Hae* III, *Hin* fI, and *Sau* 3A. Restriction maps were compared by computer analysis (Tavtigian 1992). Where a mRNA species was represented by more than one clone, only the longest corresponding cDNA was used for further analysis. cDNAs from this level of analysis were combined for use in the backprobe [see step 4]. The mid-G1 library was screened through six rounds of six plates each, while the growth arrested library was screened for two rounds of six plates.

An important practical issue was to reduce labor by eliminating redundant clones as early in the process as possible. At the level of initial plaque screens, we reduced the repeated selection of cDNAs derived from a few prevalent and differentially expressed mRNAs. As indicated in Figure 1, this was done by proceeding through several successive rounds of library screening, making triplicate lifts from each plate. The differential probes were hybridized to the first and second lifts, whereas a backprobe, generated by pooling cDNAs already obtained in earlier rounds of screening, was hybridized to the third lift; only plaques yielding differential signals on the first and second lifts and scoring negative on the third lift were selected. To further reduce the number of redundant clones maintained after a given round of screening, restriction maps were generated from the 3′ ends of the newly selected clones and a computerized comparison of the new clone restriction maps with the existing clone maps was used to identify and omit redundant clones. Plasmids bearing potentially unique cDNAs were automatically subcloned (Palazzolo *et al.*, 1990). The resulting plasmid miniprep DNAs were sequenced from their 5′ ends, yielding 250 to 325 bp of sequence from each clone. Sequences were searched against the Genbank and EMBL sequence libraries using the program BLAST (Altschul *et al.*, 1990). Remaining redundant cDNAs identified during the database searches, usually either short or internally initiated copies of highly expressed mRNAs, were eliminated at this stage.

Characterization

1) cDNA bearing plasmids were excised from primary λ clones by a cre-lox recombination system (Palazzolo *et al.*, 1990). Where more than one cDNA was obtained from the primary plaque, they were all maintained for further analysis. 2) cDNAs were Sanger-sequenced from their 5′ ends. 3) Resultant sequences were used to generate restriction maps as well as to search for sequence homologies in GENBANK and EMBL using the program BLAST (Altschul *et al.*, 1990). 4) A 5′ end fragment from each cDNA was subcloned into a dual polymerase promoter plasmid to generate a riboprobe template. 5) The expression timecourse of each RNA in serum stimulated NIH 3T3 cells was checked by ribonuclease protection (Zinn *et al.*, 1983). 6) Expression of those RNAs subject to serum regulation with mid-SR kinetics was further characterized in the conditional myc-expressing cell lines 3-5B and MER #6.

PCR cloning

Probes for the transcripts prothymosin α and Max/myn were cloned by amplification from first strand cDNA. PCR primers were the following

prothymosin α 5′: CGC GAA TTC ATG TCA GAC GCG GC[A/C/G/T] GT[A/C/G/T] GA
prothymosin α 3′: CGC GGA TCC CTG CTT CTT GGT [C/T]TC [A/C/G/T]AC [G/A]TC
Max/myn 5′: GCA GAT CTG CCA CCA TGA GCG ATA ACG ATG ACA T[C/T]G A[G/A]G T
Max/myn 3′: GCG TCG ACT CAG CTG GCC TCC ATC CGG A[G/A][C/T] TT[C/T] TT

The Prothymosin α PCR product was cloned as an *Eco* RI-*Bam* HI fragment into a dual promoter vector to serve as a riboprobe template. A *Bgl* II (PCR generated site)-*Sma* I fragment from the 5′ end of Max/myn was subcloned for the same purpose.

Other Riboprobe Templates

Several other plasmids were prepared for use as riboprobe templates in the work. pmyc1-HA contains a 122 bp *Hind*III-*Alu* I fragment from the first exon of c-myc. The *Hind*III site is located between P1 and P2 (Bernard *et al.*, 1983) within the c-myc promoter. pmyc2-XR contains a 192 bp *Xba* I-*Eco* RV fragment starting near the 3′ end of the first intron of c-myc and extending into the second exon. pMHS-SX contains a 646 bp *Sma* I-*Xba* I internal fragment from the mouse HSP 70 cDNA MHS 214 (Lowe and Moran, 1986). pfos-PP2 contains a 108 bp *Pvu* II-*Pst* I fragment spanning the 5′ end of the c-fos transcript (Van Beveren *et al.*, 1983). pp53-XA contains a 1005 bp *Xho* I-*Asp* 718 fragment from the p53 tumor antigen gene (Tan *et al.*, 1986). pβ5 contains an approximately 135 bp 3′ end fragment from a mouse tubulin β5 cDNA (Lewis *et al.*, 1985) from which the poly-A tract has been trimmed with exonuclease Bal 31.

RNA Measurement

RNA probes were synthesized using T7, T3, or SP6 RNA polymerase essentially as recommended by the enzyme manufacturers. Full length probes were excised from 5% acrylamide gels crosslinked with BAC (*N,N′*-bis-acrylylcystamine, Bio-Rad, Richmond, CA) and the acrylamide was dissolved with 200 mM 2-mercaptoethanol in TE. RNase protections were performed by a modification of the procedure described by Zinn *et al.* (Zinn *et al.*, 1983). One or two micrograms of cellular RNA plus sufficient tRNA carrier to make an RNA total of 25 μg were coprecipitated with probe. Samples were redissolved in 30 μl of RNA hybridization buffer (40 mM piperazine-*N,N′*-bis(2-ethanesulfonic acid [PIPES] pH 6.7, 400 mM NaCl, 1 mM EDTA, 80% formamide), denatured at 80° for 10 min, and hybridized for >12 h. Probe concentrations and hybridization temperatures were optimized so that hybridizations proceeded to at least 90% of completion in 12 h. Hybridizations were digested with the addition of 300 μl of 1500 units/ml RNase T1 (BRL) in RNase digestion buffer (10 mM Tris pH 7.5, 300 mM NaCl, 5 mM EDTA) for 60 min at 30°, followed by 200 μl of 250 μg/ml proteinase K, 1% sodium dodecyl sulfate (SDS) for 15 min at 30°. Handling of samples containing dissolved BAC acrylamide was facilitated by co-precipitating RNA samples and probe out of 100 mM 2-mercaptoethanol and using silanized tubes. Samples were prepared for electrophoresis and fractionated on 5% denaturing acrylamide gels, which were then dried and exposed to X-ray film. Count per minute data were obtained from the dried gels on a phosphoimager (Molecular Dynamics, Sunnyvale, CA) and converted to transcript per cell data using internal CPM standards.

DNA Replication Assays

For experiments with NIH 3T3 and 3-5B cells, Polylysine/fibronectin coated 24 well plates were seeded at a density of 2×10^4 cells per well in 0.5 ml of DMEM plus 10% serum. Twelve hours later the cells were washed with PBS and fed with 0.75 ml of DMI. Twelve hours later the cells were refed with 0.75 ml of DMI. Twenty four hours later the cells were zinc induced with the addition of various concentrations of zinc sulfate in DMEM phosphate free HEPES, mock induced with the addition of an appropriate volume of DMEM phosphate free HEPES, or serum induced with the addition of FBS to 5% final concentration. For experiments with Balb/c 3T3 and MER #6 cells, fibronectin-coated 24 well plates (Collaborative Research, Bedford, MA) were seeded at a density of 3×10^4 cells per well in 0.5 ml of phenol red free DMEM that had been adjusted to 4 mM HEPES with the addition of dry HEPES free acid, 5% calf serum, 1× glutamine/pen/strep, and 1× GMS-X medium supplement. Twenty-four hours later the cells were refed with 0.75 ml of the same medium. Seventy-two hours later the cells were β-estradiol induced with the addition of various concentrations of β-estradiol in phenol red free DMEM, mock induced with the addition of an appropriate volume of phenol red free DMEM, or serum induced with the addition of FBS to 15% final concentration. In both procedures, entry into S-phase was monitored by adding 37.5 μl of a labeling mixture containing 5-bromodeoxyuridine and 5-fluorouracil (Amersham, Arlington Heights, IL) 14 h after stimulation. Experiments were terminated 10 h after labeling mix addition by washing the cells with PBSA and then fixing them in 5% acetic acid in ethanol. Immunoperoxidase staining employed the Amersham cell proliferation kit. Wells were washed three times for 3 min with PBS, incubated with eightfold diluted anti-BrdU mAB/nuclease mix for 4 h at room temperature, washed three times for 3 min with PBS, incubated with fourfold diluted peroxidase labeled anti-mouse Ab for 2 h at room temperature, washed three times for 3 min with PBS, stained with nickel/cobalt enhanced DAB, and counterstained with fast green FCF.

Western Blots

Polylysine/fibronectin coated 6-cm plates were seeded at a density of $2.5–4.0 \times 10^5$ cells per plate in DMEM plus 10% calf serum. Twelve hours later cells were washed with PBSA and fed with 3 ml of DMI. Twelve hours later cells were refed with 3 ml of DMI. Twenty-four hours later cells were induced with 300 μl of 3:1 DMEM:F12, 150 μl of fetal bovine serum (Hazleton Biologics, Lenexa, KS), or 300 μl of 220 μM $ZnSO_4$ in 3:1 DMEM:F12. Cells were harvested over a 16-h timecourse as indicated in the Figure 4 legend. Cells were lysed in 200 μl of loading buffer (80 mM Tris pH 6.8, 100 mM dithiothreitol, 2% SDS, 10% glycerol). Cell lysates were fractionated on acrylamide SDS gels and electroblotted to Nitroplus 2000 (Micron Separations, Westboro, MA). Filters were blocked for 2 h at room temperature in 1× Tris-buffered saline (TBS), 0.5% Tween 20, 2% BSA. Antibody incubations were in the same solution with the addition of 2 μg/ml anti-myc monoclonal antibody H60C37 ^{125}I-labeled to a specific activity of ~100 Ci/mM. Antibody incubations were for 12 h, followed by five 20-min washes in blocking solution.

Nuclear Run-on Assays

For nuclear run-ons, MER #6 cells were brought to growth arrest by contact inhibition in phenol red free DMEM that had been adjusted to 4 mM HEPES with the addition of dry HEPES free acid, 5% calf serum, 1× glutamine/pen/strep, 1× GMS-X medium supplement (Gibco), and 100 μg/ml G418 (Gibco). Preparation of nuclei and transcription reaction conditions were modified from Greenberg and Bender (1990). Cells from six 15-cm plates (~2×10^7 cells/plate) were washed twice with PBS, scraped with a rubber policeman, and centrifuged for 5 min at 500 × g at 4°. All subsequent steps were carried out at 4°. Supernates were removed and cells resuspended in 40 volumes of lysis buffer (10 mM Tris pH 7.4, 3 mM $CaCl_2$, and 2 mM $MgCl_2$). Cells were centrifuged for 5 min at 500 × g, supernates were removed, and cells were resuspended in 2 ml of lysis buffer followed by the addition of 2 ml of lysis buffer plus 1% NP-40. The preparations were transferred to a dounce and remaining intact cells broken by 12 strokes of the homogenizer. The lysates were spun at 500 g for 5 min, supernates were removed, and nuclei resuspended in 400 μl of glycerol buffer (50 mM Tris pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA).

Fresh nuclear preparations were mixed with 400 μl of 2× reaction buffer (10 mM Tris pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 5 mM DTT,

and 1 mM each ATP, CTP, and GTP) containing 300 μCi of α-^{32}P UTP at 800 Ci/mM and 40 units of RNasin. Reactions were incubated for 20 min at 30°. One and one-fifth microliters of HSB (10 mM Tris pH 7.4, 500 mM NaCl, 50 mM $MgCl_2$, and 2 mM $CaCl_2$) was then added to each reaction, and DNA in the reaction mixtures was digested with 100 units of DNase I (RQ1, Promega, Madison, WI) for 15 min at 30°. Four hundred milliliters SDS/Tris buffer (500 mM Tris pH 7.4, 5% SDS, and 125 mM EDTA) was added, and the reactions were digested with 400 μg of proteinase K for 30 min at 42°. The RNA preparations were then extracted with phenol chloroform and isopropanol precipitated. Pellets were redissolved in 1 ml of DNase buffer (20 mM HEPES pH 7.5, 5 mM $MgCl_2$, and 1 mM $CaCl_2$), incubated for 10 min at 37°, and redigested with 50 units of RQ1 DNase for 30 min at 37°. The DNase reactions were stopped with the addition of EDTA to 15 mM and SDS to 0.9%, digested with 60 μg of proteinase K for 30 min at 37°, and phenol chloroform extracted. The RNA preparations were denatured with 250 μl of 1 M NaOH for 10 min on ice, neutralized with 500 μl of 1 M HEPES (free acid), and isopropanol precipitated.

The RNA pellets were redissolved in 1 ml of TES solution (10 mM TES [N-tris(hydroxymethyl)methyl-2-aminoethansulfonic acid] (Sigma) pH 7.4, 10 mM EDTA, and 0.2% SDS) for 30 min at RT, mixed with 1 ml of TES/NaCl (TES solution plus 600 mM NaCl), and hybridized for 60 h at 65° to linearized, denatured plasmid DNAs that had been dot-blotted and UV crosslinked onto Hybond-N strips (Amersham). Plasmids used were pI-8-10B (LDH), pI-8-29, and pI-8-36 (ODC) (obtained from differential screening of the mid-G1 serum stimulated cDNA library), and pmyc A, a full length c-myc cDNA that was obtained during initial characterization of the mid-G1 serum stimulated cDNA library. Filters were washed twice in 2× SSC for 60 min at 65°, digested with RNase A at 100 μg/ml in 2× SSC for 30 min at 37° and washed for an additional hour in 2× SSC at 37°. The filter strips were then air dried and exposed on a phosphoimager (Molecular Dynamics).

RESULTS

Cloning Strategy

The initial goal in this work was to identify mid-SR class genes. cDNA libraries were prepared from both serum starved and 8-h serum stimulated Balb/c 3T3 cells. These libraries were screened with probes enriched for sequences either induced or repressed in response to mitogen stimulation. Figure 1 presents an outline of the cloning strategy (see MATERIALS AND METHODS for a detailed description). The design was influenced by several considerations. First, we focused the selection scheme on the kinetics of progression through G_1 following emergence from G_0. In this experimental regime, the 8-h timepoint is reproducibly within G_1, close to the restriction point (R-point), beyond which entry into S-phase becomes insensitive to inhibition by serum removal (Pardee, 1989). Thus, execution genes whose new transcription and translation are needed for progression from G_1 to S should be well-represented at the 8-h point (entry into S under these conditions is at ~14 h). The 8-h point was also expected to allow for the decay of prominent G_0 transcripts derived from genes that are down-regulated in response to serum. We will refer to transcripts differentially regulated at 8 h as mid-serum response, or mid-SR, to distinguish them from a potentially distinct class regulated in mid-G_1 of exponentially growing cells. A second strategic consideration was that

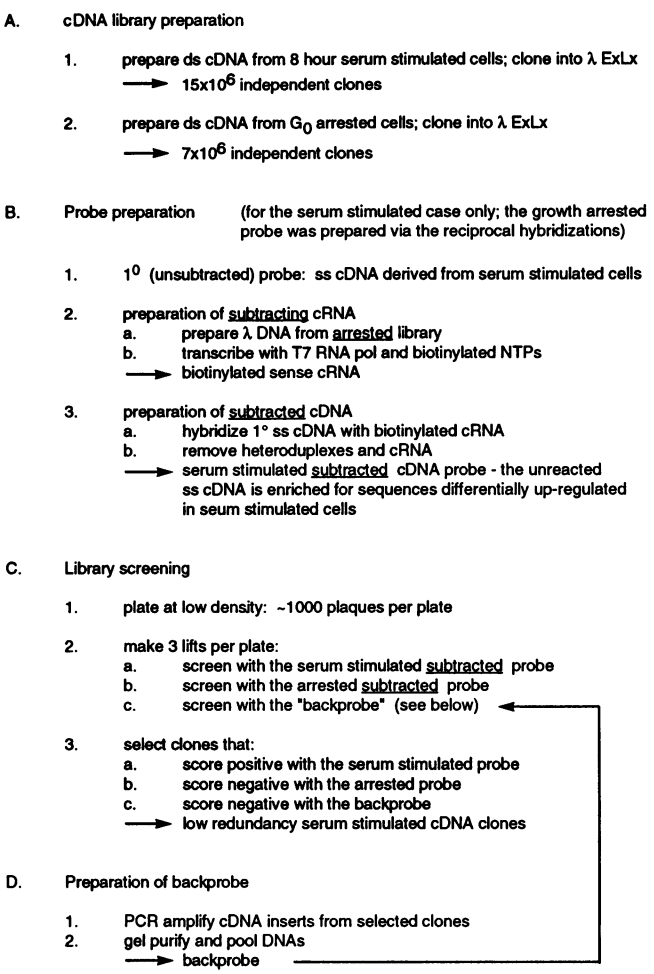

Figure 1. Flow chart of the cDNA library screening strategy.

we wanted to obtain clones corresponding to mRNAs of all prevalence classes. This was achieved by using subtracted hybridization probes enriched in differentially expressed sequences, which effectively increased the screening sensitivity for lower abundance cDNAs (Sargent, 1987; Palazzolo *et al.*, 1990). Third, we anticipated that some genes whose expression is initiated early, qualifying them as members of the immediate early kinetic class, will continue to be expressed through G_1, and that this later phase of expression may also identify them as mid-SR class genes. c-Myc itself, for example, displays such a pattern of expression (Muller *et al.*, 1984; Lau and Nathans, 1987). While there are several different possibilities for the regulatory basis of such expression, we wanted to detect such genes in the initial screen. For this reason, we did not initially eliminate all immediate early class genes but later identified them as a kinetic subset.

The growth arrested and serum stimulated cDNA libraries contained 7×10^6 and 1.5×10^7 clones, respectively. Because the fibroblasts used in this work contain

in the range of 3–10 × 10^5 mRNA molecules per cell (Jendrisak *et al.*, 1987), the libraries are large enough to ensure that even the rarest mRNA species are represented, except for possible exceptional sequences that may have been peculiarly poor substrates for reverse transcription. Although the libraries are comprehensive, the screening reported here was not extensive enough to be saturating and was more complete for the serum induced case than for the repressed case. The serum stimulated library was screened through six rounds of six plates each (at ~1000 plaques per plate), and the growth arrested library was screened through two rounds of six plates each (see MATERIALS AND METHODS for details of screening and clone management). From 100 differentially hybridized plaques ~40 cDNAs were found to be nonredundant by the criteria of 3′ end restriction mapping and limited 5′ end sequence analysis. These were subjected to further analysis.

Expression in Growth Arrested and Serum Stimulated Cells

The 40 candidate 8-h response clones were tested for the kinetics of their response to serum. Members of the immediate early kinetic class were identified and separated from those clones whose RNAs were most strongly differentially regulated at the 8-h mid-G_1 point. For operational purposes, we defined mid-SR regulated clones as those either 1) expressed at higher levels 8 h following serum stimulation than 1 h following or 2) repressed during G_1 following serum stimulation of quiescent cells. To confirm magnitude and timing of regulation, ribonuclease protection assays were used. RNAs used for screening were from NIH 3T3 cells that had been brought to growth arrest in defined medium, then mock or serum induced for 1 or 8 h. In addition, each clone was tested for expression in asynchronous exponentially growing cells. RNA from NIH 3T3 cells was used in rescreening to filter out any clone whose response was particular to the Balb/c 3T3 line used to construct and screen the original libraries. Of the 40 unique clones obtained from initial screens, 18 were classified as mid-SR genes; 15 of these were induced (Figure 2B) and 3 were repressed (Figure 2C) by serum treatment. The remaining clones were either subject to stimulation by serum with predominantly early G_1 kinetics or were not reproducibly regulated. Clones expressed most highly at 1 h have not yet been subscreened to differentiate those that are regulated independently of protein synthesis (the criterion that distinguishes the immediate early class) from those that are dependent on immediate early products. Nomenclature: serum stimulated clones were initially named I-8-x where I-8 indicates serum induced at 8 h and x is the number assigned to the primary plaque upon its selection. Repressed clones were named R-8-x where R-8 indicates serum repressed at 8 h. Where an original phage plug later proved to contain more than one cDNA clone, the clone number is followed by a letter, i.e., I-8-10A and I-8-10B. DNA sequencing was performed to determine possible identities of the cDNAs. Several proved to be known genes or their near relatives, while others were not detectably homologous to sequences in GENBANK. Because our screening was not saturating, we expect that there are many other mid-SR cDNA species that remain to be identified. A conceptually similar search for genes expressed in this pattern was recently reported (Lanahan *et al.*, 1992), and it identified 13 delayed early response genes that are not overlapping with those reported here.

For comparison of mid-SR genes with other work, panel A of Figure 2 presents the classic immediate early transcripts c-fos and c-jun (Muller *et al.*, 1984; Lau and Nathans, 1987; Almendral *et al.*, 1988), as well as the additional recently cloned genes of potential interest, Max/myn and Id (Benezra *et al.*, 1990; Blackwood and Eisenman, 1990; Prendergast *et al.*, 1991). The serum induced I-8 clones (panel B) and serum repressed R-8 clones (panel C) correspond to mRNAs from all prevalence classes, as anticipated from the cloning strategy. For example, I-8-16 (cytoplasmic [A-X] actin), R-8-1 (alpha-1 [3] collagen), and R-8-9 (translation elongation factor 1) were all present at more than 1000 transcripts per cell. At the other extreme, I-8-23, I-8-29, and I-8-30B were present at 50 or fewer transcripts per cell at the 8 h serum induction timepoint. The degree of regulation varied from a reproducible but modest 2-fold induction of I-8-30B to more than 100-fold stimulation of I-8-1 (tenascin) and 50-fold repression of R-8-1 (alpha-1 [3] collagen). In asynchronous exponentially growing cells, most species tested were expressed at levels well below their peak in serum stimulated G_1 (for the I-8 series) or G_0 (for the R-8 series). The exception was I-8-41 (heat stable antigen, HSA) (Kay *et al.*, 1990), which was more highly expressed in cycling cells than at any point tested during emergence from G_0.

Regulatory Capacity of Conditional myc Expression

To screen the mid-SR clones for myc responsiveness, we used an NIH 3T3 cell line (3-5B) that expresses c-myc driven by the metallothionein-I promoter (Tavtigian, 1992). This cell line is subject to growth arrest in defined medium, as is the parental NIH 3T3 line. As in other conceptually similar cell lines with inducible myc activity (Cavalieri and Goldfarb, 1987), ectopic c-myc expression under arrest conditions is sufficient to stimulate a subpopulation of the growth arrested cells (50–60%) to reenter the cell cycle, traverse G_1, and enter S-phase (Figure 3). During the course of such an experiment, c-myc protein peaks at levels exceeding those present in serum stimulated NIH 3T3 cells by 5- to 10-fold (Figure 4). A second cell line used to verify myc responsiveness was MER #6, a Balb/c 3T3 based cell

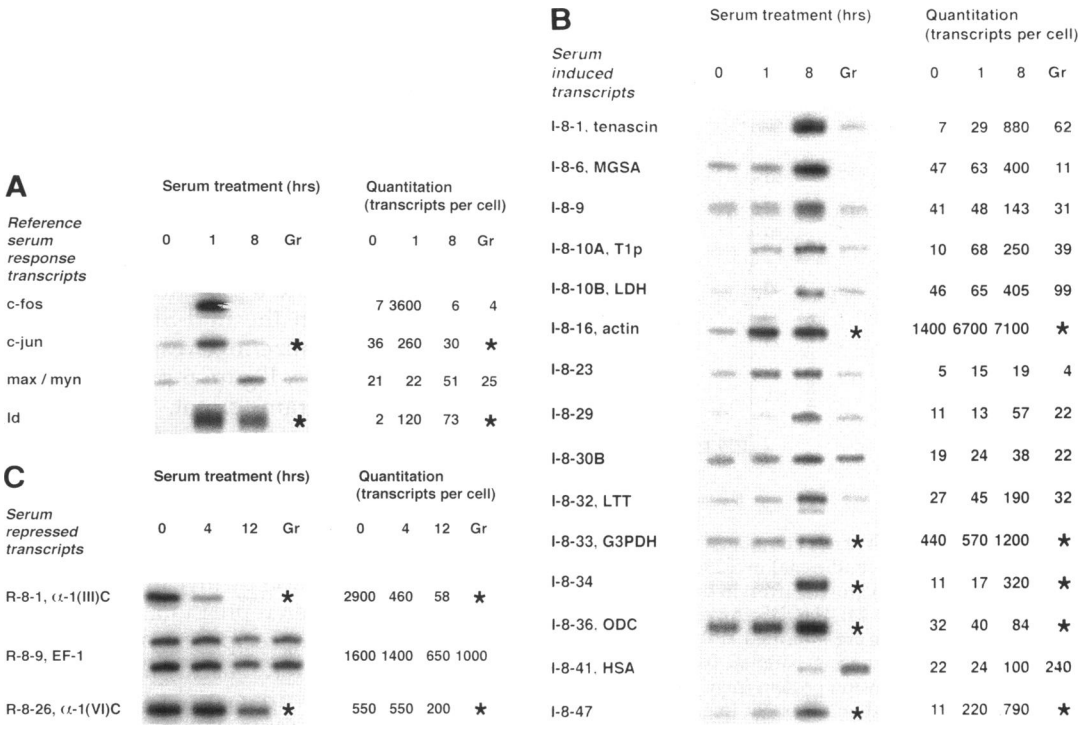

Figure 2. Expression of cloned transcripts regulated in NIH 3T3 cells with mid-SR kinetics. NIH 3T3 cells were seeded and brought to growth arrest in defined medium as indicated in MATERIALS AND METHODS. Arrested cells were mock or serum stimulated. RNAs generated from mock, serum stimulated, and exponentially growing cells were analyzed by ribonuclease protection. Quantitative data from each set of ribonuclease protections were obtained from the dried gels on a phosphoimager (Molecular Dynamics). Using internal standards on each gel, transcripts per cell was calculated as:

$$\frac{\text{protected cpm } (\mu\text{g total RNA}^{-1})}{\text{protected probe activity, cpm mol}^{-1}} \times \frac{1\ \mu\text{g total RNA}}{50{,}000\ \text{cells}} \times 6.02 \times 10^{23}\ \text{molecules mole}^{-1}$$

corrected for probe decay over the course of the experiment.
More accurately, this is a measure of *transcripts per 20 pg of total RNA;* in our experience, a reasonable estimate of the RNA content of a growth arrested 3T3 cell.

(A) Reference serum response transcripts. (B) Serum induced transcripts. (C) Serum repressed transcripts. Timecourse: 0, mock induced; 1, 4, 8, 12, serum treated for 1, 4, 8, or 12 h; Gr, exponentially growing cells. Abbreviations: α-1(III)C, α-1 type III collagen; EF-1, translation elongation factor 1; α-1(VI)C, α-1 type VI collagen; MGSA, melanoma growth stimulatory activity; T1p, T1 protein; LDH, lactate dehydrogenase; LTT, liver thiol transferase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ODC, ornithine decarboxylase; HSA, heat stable antigen. * Indicates that a particular measurement was not made.

line expressing a c-myc/estrogen receptor fusion protein (Eilers *et al.*, 1989, 1991). Fifty to sixty percent of MER #6 cells reenter the cell cycle following hormonal activation of the fusion protein (Eilers *et al.*, 1991; see also Figure 3).

To calibrate RNA responses by the selected mid-SR genes to conditional c-myc we used several already known G_1-regulated transcripts (the immediate early genes c-fos and endogenous c-myc, and the mid-SR gene p53 tumor antigen; tubulin β5 serves as an unaffected control) (Figure 5). In both parental 3T3 cells and myc23c expressing 3-5B cells, c-fos RNA was present at very low levels and did not vary significantly with c-myc expression. As shown previously (Eilers *et al.*, 1991), the immediate early burst of c-fos RNA synthesis following serum stimulation is bypassed in a c-myc stimulated emergence from G_0. Endogenous c-myc RNA levels were chronically repressed in 3-5B cells relative to 3T3 cells; this was probably a consequence of basal c-myc transgene expression in 3-5B cells. In addition, the endogenous c-myc transcript was further repressed over the timecourse of the myc transgene induction. That myc itself shows different regulation in response to full serum stimulation (up-regulation at mid-G_1 relative to G_0) compared with the response to ectopic transgene myc (down-regulation relative to G_0), raises the possibility that other genes from the mid-SR panel might display disparate responses to the mitogen- versus myc-driven $G_0 \rightarrow S$ experimental regimes.

p53 Transcript levels remained steady in control 3T3 cells treated with zinc, but they were induced 2- to 3-fold between 4 and 12 h after zinc addition to 3-5B

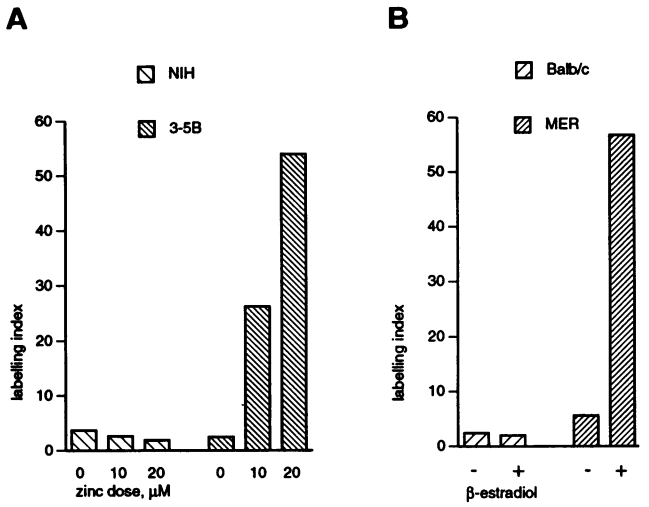

Figure 3. Emergence from growth arrest stimulated by conditional myc expression. (A) NIH 3T3 and conditional myc23c expressing 3-5B cells were seeded and brought to growth arrest in defined medium as indicated in MATERIALS AND METHODS. Arrested cells were mock or zinc stimulated; zinc doses are indicated at the column bottoms. (B) Similar experiments comparing parental Balb/c 3T3 cells with the c-myc estrogen receptor fusion protein (myc ER) expressing cell line MER #6. Cells were seeded and brought to growth arrest in medium containing 5% calf serum as indicated in MATERIALS AND METHODS. Arrested cells were mock or β-estradiol (100 nM) stimulated as indicated at bottom of each column. Cells were labeled with BrdU from 14 to 24 h following stimulation. Fixation and immunoperoxidase staining were as in MATERIALS AND METHODS. Approximately 400 cells were counted for each zinc dose; labeling index is the number of peroxidase stained cells divided by the total number of cells.

cells, consistent with its timecourse of up-regulation in response to serum stimulation (Reich and Levine, 1984). This identifies p53 as a target, direct or indirect, of myc in this system. This result is consistent with previous observations that p53 is among the proteins that are overexpressed in cells whose growth characteristics have been altered by chronic c-myc overexpression (Shiroki *et al.*, 1986), although it has been difficult in such systems to discriminate obligate responses to myc from long term indirect effects. As discussed later, other independent molecular data argue that p53 is a good candidate for direct regulation by myc. Its response is consistent with that possibility but also suggests that the magnitude of myc response expected from unknown genes in the mid-SR panel may be quantitatively modest, as is the case for p53.

Identification of myc Regulated Transcripts Among Mid-SR Regulated Clones

The metallothionein promoted c-myc gene expressed by 3-5B cells included an upstream glucocorticoid regulated enhancer derived from MMTV. We had originally planned to use this second, metal-independent myc induction system to control for possible phenotypic effects secondary to metal treatment. Unfortunately, regulation of the myc23c transgene by glucocorticoids proved weak and inconsistent in this cellular environment and was not pursued further. Instead, we substituted the cell line MER #6, which expresses a c-myc/estrogen receptor fusion protein (myc-ER) in the Balb/c 3T3 cell background (Eilers *et al.*, 1989, 1991) (a kind gift from Sabine Schirm and J. Michael Bishop).

Table 1 and Figure 6 present the effect of conditional myc expression on the mid-SR regulated cDNAs. To more simply visualize myc response in the two conditional myc systems, Figure 6 presents data from Table 1 in a scatter diagram format. While most of the transcripts cluster around (1,1), indicative of no significant response to elevated myc expression, I-8-10B (lactate dehydrogenase), I-8-36 (ornithine decarboxylase), I-8-29 (not found in the databases), and I-8-30B (not found in the databases) all lie in the upper right quadrant, indicative of induction by myc in both regimes. To better visualize myc-driven repression, the data were replotted in Figure 6B as the reciprocal of the values in 6A. In this analysis, I-8-1 (tenascin) and I-8-41 (heat stable antigen) lie in the upper right quadrant, indicative of repression by myc. Of the 18 mid-SR regulated mRNAs,

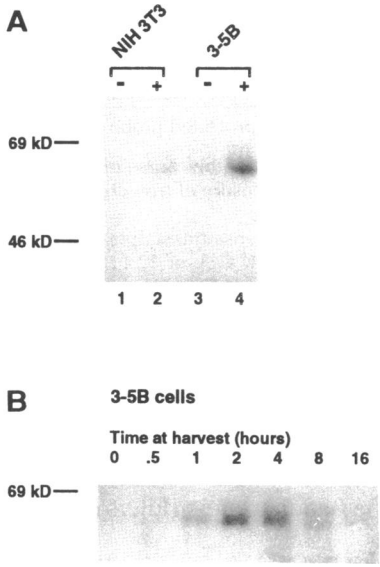

Figure 4. Western analysis of c-myc protein expression in NIH 3T3 and myc23c expressing 3-5B cells. (A) (−): cells were mock induced. (+): 3T3 cells were induced by adjusting the medium to 5% fetal bovine serum. 3-5B cells were induced by adjusting the medium to 20 μM $ZnSO_4$. All cells were harvested after 4 h of induction. (B) 3-5B cells were harvested over a 16 h timecourse following induction. Cells in the "0" timepoint were harvested 4 h after mock induction. The other samples were harvested at the times indicated at the tops of the lanes. Cells were seeded, brought to growth arrest, induced, harvested, and blotted as indicated in MATERIALS AND METHODS. The labeling antibody was ^{125}I conjugated anti c-myc monoclonal H60C37.

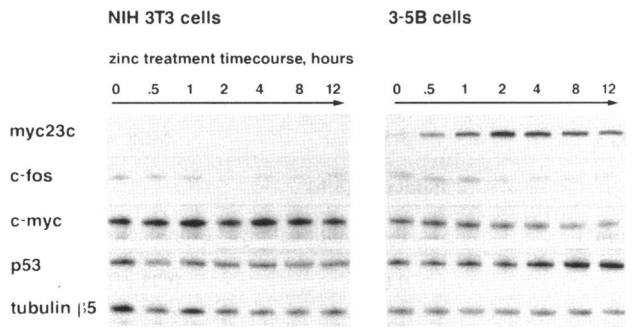

Figure 5. Comparison of selected mRNA expression in NIH 3T3 and myc23c expressing 3-5B cells. NIH 3T3 and 3-5B cells were seeded and brought to growth arrest in defined medium as indicated in MATERIALS AND METHODS. Arrested cells were either mock stimulated or treated with 20 μM zinc. RNAs generated from mock and zinc treated cells were analyzed by ribonuclease protection. Timecourse: 0, mock stimulated cells; .5→12, cells treated with zinc for 0.5 to 12 h. Please note that the ratio of the sensitivity of the c-myc to myc23c protections was 8:1; the sensitivity of the c-fos protection was equivalent to that of the c-myc protection, just sufficient to detect basal c-fos expression.

5 (lactate dehydrogenase, ornithine decarboxylase, tenascin, I-8-29, and I-8-30B) are induced or repressed more than twofold following stimulation of myc expression in one of the two systems; in each case, that same mRNA is regulated in the same manner, though not necessarily to the same degree, in the alternate myc expressing cell system. Expression from the immediate early serum response genes c-fos, c-jun, and c-myc and the previously identified myc-responsive gene prothymosin α were also measured in this experiment. Myc behaved essentially as before in 3-5B cells (Figure 5), while in MER #6 cells myc was constitutively repressed to very low levels, presumably due to basal activity from the myc-ER fusion protein (Eilers *et al.*, 1989). Neither c-fos nor c-jun responded to myc expression in either system. Prothymosin α was induced in 3-5B cells, confirming in this NIH 3T3 cellular background the original observation of myc inducibility reported in MycER expressing Rat 1 cells (Eilers *et al.*, 1991).

The RNA level comparisons between the NIH 3T3 and Balb/c 3T3 based conditional myc systems were made in the experimental condition of growth arrest at low density in serum free defined medium. Primary RNA level data for the key myc responses observed in that growth arrest condition are shown in Figure 7A. In addition, Balb/c 3T3 and MER #6 cells can also be brought to growth arrest efficiently by contact inhibition in medium containing 5% calf serum (Figure 3). The entire set of genes up-regulated by conditional myc in MER #6 cells arrested in defined medium also responded to conditional myc in this second arrest paradigm. Furthermore, most of those transcripts, especially prothymosin α, responded more strongly to conditional myc in this system than in the defined medium growth arrest system (Figure 7B).

Myc Stimulates Target Genes at the Transcriptional Level

Responses to conditional myc could be either transcriptional or post-transcriptional. If a target gene is regulated by a transcriptional mechanism, it then becomes a candidate for direct regulation by myc-containing transcription factors. Nuclear run-on assays were done to test for transcription level regulation of myc targets. Due to the limited sensitivity of nuclear run-ons, we focused on the most robustly induced myc target genes: I-8-10B (lactate dehydrogenase), I-8-29 (not found in data base) and I-8-36 (ornithine decarboxylase). Figure 8 presents the results from a representative run-on experiment in which MER #6 cells were brought to density arrest and then treated with estrogen to induce the myc-ER protein activity. After 3 h of stimulation, we consistently observed an increase of the myc regulated run-off transcripts. Quantitation was done using a phosphoimager and revealed that after 3 h of stimulation, I-8-29 increased by fivefold over the zero time point. Since I-8-10B (lactate dehydrogenase) and I-8-36 (ornithine decarboxylase) were below the threshold of detection in unstimulated cells, we could not calculate a fold-induction. However, by placing a generous maximum limit on the largest signal that would have gone undetected at the zero time point, we can estimate that stimulation was greater than or equal to 12- and 8-fold at 3 h for I-8-10B (lactate dehydrogenase) and I-8-36 (ornithine decarboxylase), respectively. Conversely, after 3 h of estrogen stimulation, myc run-off transcripts decreased by 80%, which also shows that the zero time transcription was not simply defficient in transcription from all genes. These results show that the mechanism of myc induction for I-8-29, ODC, and LDH is, at least in part, transcriptional. The observed transcriptional effects do not preclude additional, secondary post-transcriptional effects such as mRNA stabilization, but at these early kinetic points, transcription appears to account for the observed increase in RNA levels.

DISCUSSION

Differences and Similarities Between Serum-driven and myc-driven Emergence From G_0

Following stimulation of ectopic myc expression in G_0 arrested cells, both 3-5B and MER #6 cells emerge from growth arrest, progress through G_1, and enter S-phase. However, in these systems only a fraction (usually 40–50%) of the arrested cells ultimately enter S-phase (Figure 3). In contrast, >95% of these same cells will enter S-phase following serum stimulation, which suggests that there is at least a significant quantitative difference in the responsiveness of cells to myc versus serum; in principle, a substantial qualitative difference might also exist although there has been no direct evidence for this. Our results provide direct evidence for qualitative

Table 1A. cDNA clones subject to serum regulation with mid-SR kinetics

		Cell system		
		NIH3T3	3-5B	MER #6
		Induction (8 h)		
Clone	Clone identity	By serum[a]	By conditional myc[b] expression	
I-8-1	Tenascin	110	0.69	0.33 ± 0.2
I-8-6	MGSA[c]	8.6	1.0	0.66 ± 0.1
I-8-9	Not identified	3.6	1.0	0.84 ± 0.3
I-8-10A	Mouse T1 protein	26	0.9	Not detected
I-8-10B	Lactate dehydrogenase	8.9	2.21	2.0 ± 0.1
I-8-16	A-X actin	4.6	0.6	0.88 ± 0.1
I-8-23	Not identified	3.2	Not done	0.96 ± 0.3
I-8-29	Not identified[d]	5.1	1.73	5.0 ± 0.6
I-8-30B	Not identified	2.0	2.6	1.4 ± 0.2
I-8-32	Liver thiol transferase	7.0	1.1	0.86 ± 0.1
I-8-33	G-3-P dehydrogenase[e]	2.9	1.2	1.0 ± 0.1
I-8-34	Not identified	28	0.89	1.3 ± 0.7
I-8-36	Ornithine decarboxylase	2.6	3.96	1.7 ± 0.2
I-8-41	Heat-stable antigen	4.6	Not detected	0.5 ± 0.1
I-8-47	Not identified	70	1.49	1.4 ± 0.2
		Repression[f]		
		By serum at 12 h	By conditional myc expression at 8 h	
R-8-1	Alpha-1 (3) collagen	50	1.7	1.0 ± 0.1
R-8-9	Elongation factor 1	2.4	Not done	1.1 ± 0.2
R-8-26	Alpha-1 (6) collagen	2.7	Not done	1.1 ± 0.1

[a] Induction by serum: transcript prevalence in serum stimulated cells divided by transcript prevalence in growth arrested cells.

[b] Induction by conditional myc expression:

$$\frac{\text{fold change upon induction in conditional cell line}}{\text{fold change upon induction in control 3T3 cell line}}$$

[c] MGSA: melanoma growth stimulatory activity.

[d] Shares some sequence homology with methylenetetrahydrofolate synthetase.

[e] G-3-P dehydrogenase: glyceraldehyde-3-phosphate dehydrogenase.

[f] Repression values are the reciprocal of induction values.

Accession numbers: I-8-1 (D90343); I-8-6 (X12510); I-8-9 (U06663); I-8-10A (M24843); I-8-10B (M27554); I-8-16 (J04181); I-8-23 (U06664); I-8-29 (U06665); I-8-30B (U06666); I-8-32 (M31453); I-8-33 (M32599); I-8-34 (U06667); I-8-36 (M10624); I-8-41 (M58661); I-8-47 (U06668); R-8-01 (X06700); R-8-26 (J04598).

differences as well as quantitative ones, and suggest that myc driven progression is not merely a subset of the events that occur in response to serum. Divergence between serum- and myc-driven G_1 phases was revealed in the cases of endogenous myc and tenascin (I-8-1), an extracellular matrix protein (for review, see Sage and Bornstein, 1991). Both were upregulated by serum but downregulated by conditional myc expression. The differential response of tenascin to myc versus serum may have functional implications for the limited oncogenic effects of deregulated myc, by itself, in fibroblast and mesenchymal cell types, as tenascin upregulation has been associated with tumor progression (Inaguma *et al.*, 1988). Thus efficient growth transformation of such cells normally requires collaboration of myc with activated Ras or additional growth factors (Bishop, 1986). It will therefore be interesting to test whether ectopic tenascin expression results in progression of myc immortalized cells to a more aggressively transformed, tumorgenic state without additional input from activated Ras.

Comparison of the serum response with myc response also showed that many genes regulated by serum do not respond detectably to myc. In some cases, these differences are striking in their magnitude [T1 protein (I-8-10A) and I-8-34]. Recently gained knowledge about the components of the immediate early regulatory class provides a possible explanation. Some prominent

Table 1B. cDNA clones subject to serum regulation with mid-SR kinetics

	Induction (8 h)		
	By serum[a]	By conditional myc[b] expression	
	Cell system		
	NIH 3T3	3-5B	MER #6
Alpha prothymosin	1.7	3.3	1.2 ± 0.1
c-Myc	15	0.36	0.76 ± 0.4
Max/myn	2.4	2.1	1.2 ± 0.3
p53 Tumor antigen	3.8	2.3	1.2 ± 0.2
c-fos	0.83	0.96	Not done
c-jun	0.80	0.79	Not done
fra-1	8.5	1.0	Not done
HSP 70 MHS 214	3.0	1.2	Not done
Id	31	0.89	Not done

[a] Induction by serum: transcript prevalence in serum stimulated cells divided by transcript prevalence in growth arrested cells.

[b] Induction by conditional myc expression:

$$\frac{\text{fold change upon induction in conditional cell line}}{\text{fold change upon induction in control 3T3 cell line}}$$

members of the immediate early serum response are not at all induced by ectopic myc; rather, in a myc driven emergence from G_0 regulators such as c-fos and c-jun are apparently bypassed (Eilers *et al.*, 1991; Yang *et al.*, 1991; see also Figure 5 and Table 1). It therefore seems likely that some mid-SR class genes would depend heavily or entirely on the bypassed regulators, and little or not at all on c-myc. Even the limited panel of mid-SR genes presented here includes a dozen that are good candidates for regulation by non-myc immediate early regulators.

At the cellular level, a straightforward conclusion is that myc-insensitive mid-SR products are not essential for progression to S-phase, although they may very well account for differences in the character and quantitative success of the full mitogen response compared with the myc response. However, viewed from a different perspective, a second and nonexclusive possibility is that some mid-SR genes found to be absent from the myc response class actually contribute to myc induced cell cycle progression by their absence. These might include regulators that are important because they normally function as checks to limit progression toward S-phase in a full serum response. Finally, the observed differences between the myc-responsive set and the larger serum responsive group point to another possibility that our experiments did not address; there may be genes that are regulated by myc in the absence of serum that are not regulated at all by serum. In the two step screen used here, these would have been absent from the starting pool. A direct plus/minus myc subtractive screen will be needed to find them.

Genes that responded similarly to myc and to serum were lactate dehydrogenase (I-8-10B), I-8-29, ornithine decarboxylase (I-8-36), and p53 tumor antigen. These similarly affected products are candidates for proteins obligatorily involved in the regulation or execution of any successful progression from G_0 to S-phase. This expectation is supported by the recent reports that forced expression of ODC in 3T3 cells is tumorgenic (Auvinen *et al.*, 1992; Moshier *et al.*, 1993).

Transcriptional Responses to Conditional myc

Nuclear run-on experiments showed that several myc-induced genes are responding transcriptionally. In this connection, it is interesting that the promoter regions of lactate dehydrogenase, ornithine decarboxylase, and

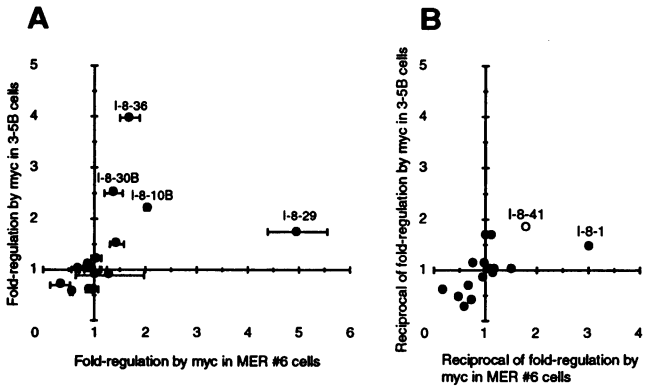

Figure 6. Scatter diagram analysis of mid-SR regulated transcript expression in conditional myc expressing 3-5B and MER #6 cells. Data are from Table 1. (A) Data for each transcript are plotted on a scatter diagram. The X-value for each point is

$$\frac{\text{fold change upon induction in MER \#6 cells}}{\text{fold change upon induction in Balb/c 3T3 cells}}.$$

The Y-value for each point is

$$\frac{\text{fold change upon induction in 3-5B cells}}{\text{fold change upon induction in NIH 3T3 cells}}.$$

The Balb/c MER #6 experiment was iterated 3 times; the plotted data are average values and the X-error bars delimit ± SD for these data. Clone numbers are provided for those transcripts that were clearly upregulated by conditional myc expression. (B) Data for each transcript are replotted on a scatter diagram where the X- and Y-values are the reciprocals of the values in part A. Clone numbers are provided for those transcripts that were clearly downregulated by conditional myc expression. Where a myc-independent transcript was either not measured or not detected in 1 of the 2 cell systems (see Table 1), its data point is placed on 1 of the axes using the fold-regulation value measured in 1 system only. The transcript I-8-41 (heat stable antigen) is not expressed in the specific NIH 3T3 isolate used to construct 3-5B cells. However, the transcript is reproducibly repressed by conditional myc expression in the Balb/c MER #6 system. Therefore, its data point is arbitrarily placed on the main diagonal at the value determined in the Balb/c MER #6 system.

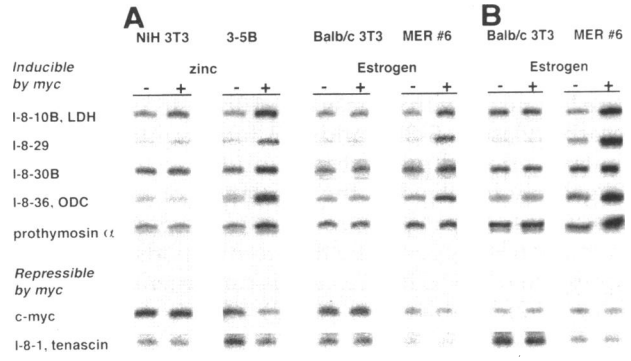

Figure 7. Expression of selected myc regulated transcripts. (A) NIH 3T3, myc23c expressing 3-5B, Balb/c 3T3, and mycER expressing MER #6 cells were seeded and brought to growth arrest in defined medium as indicated in MATERIALS AND METHODS. NIH 3T3 and 3-5B cells were mock or zinc treated; Balb/c and MER #6 cells were β-estradiol treated. (B) Balb/c 3T3 and MER #6 cells were seeded and brought to growth arrest by contact inhibition in medium containing 5% calf serum as indicated in MATERIALS AND METHODS. In both experimental regimes, cells were harvested after 8 h of stimulation. RNAs were analyzed by ribonuclease protection. The induced zinc condition was 20 μM for all transcripts except for I-8-29, for which it was 15 μM. The induced β-estradiol condition was 100 nM in all cases. Abbreviations: LDH, lactate dehydrogenase; ODC, ornithine decarboxylase.

p53 tumor antigen all contain potential myc binding sites as previously defined by in vitro target site selection experiments (Blackwell *et al.*, 1990; Halazonitas and Kandil, 1991; Kerkhoff *et al.*, 1991). We have recently shown that the myc-type E-box within the LDH promoter is a good in vitro binding site for myc:Max/myn heteromers or Max/myn:Max/myn homo-oligomers (Tavtigian, 1992). Also, a potential myc binding site within the mouse p53 promoter, located just downstream of the cap site, is a positive regulator of p53 expression (Ronen *et al.*, 1991; Reisman *et al.*, 1993). Furthermore, recent work has also demonstrated that c-myc transactivates ODC promoter-reporter constructs through an E-box inintron I of the ODC gene (Bello-Fernandez *et al.*, 1993). These genes may therefore be directly regulated by a c-myc:Max/myn heteromer in its role as a transcription factor or by Max/myn homo-oligomers, although other transcription factors, notably USF/MLTF and TFII-i (Carthew *et al.*, 1985; Gregor *et al.*, 1990; Roy *et al.*, 1991), can recognize the same core sequence and their involvement has not been ruled out. It will now be crucial to understand in detail the balance between levels of myc and Max/myn hetero- and homo-oligomers and how these levels correlate with the responses to myc and serum reported here. A relevant observation from this work was that induction of ectopic myc in 3-5B cells results in up-regulation of Max/myn RNA, so that active myc:Max/myn heteromers are probably able to form.

Other myc Target Genes

Several prior studies have been directed at identification of myc regulatory targets, and some of these targets were screened in the course of this work for responsiveness in the $G_0 \rightarrow$ S-phase inducible system. Chronic overexpression of myc leads to transcriptional repression of c-myc and to repression of MHC class I transcripts (Bernards *et al.*, 1986; Cleveland *et al.*, 1988; Versteeg *et al.*, 1988; Grignani *et al.*, 1990; Penn *et al.*, 1990). We found here that myc autorepression is also observed upon short-term myc overexpression. In constitutive myc overexpressing lines, Prendergast and Cole were able to identify two myc regulated clones, MR1 (plasminogen activator inhibitor 1), and MR2. At the mechanistic level, their data suggested that regulation was post-transcriptional in both cases (Prendergast *et al.*, 1990). Yang *et al.*, (1991) identified three collagen transcripts transcriptionally repressed by c-myc overexpression, and Eilers *et al.*, (1991) found that prothymosin α is transcriptionally induced by a c-myc/estrogen receptor fusion protein in a Rat-1 cell background, though not in a Balb/c 3T3 cell background (Eilers *et al.*, 1991; Yang *et al.*, 1991, Hevezi and Bishop, personal communication). Here we found prothymosin α to be myc-responsive in a manner that varied according to cell background and growth arrest paradigm. We therefore believe it belongs in the group that are not essential for progression but are nevertheless myc responsive in some cellular environments. Recently, several cyclins have been tested for their responses to myc (Jansen-Durr *et al.*, 1993; Tavtigian, Zabludoff, and Wold, unpublished observations). These are particularly interesting because a large body of evidence identifies the cyclins as pivotal elements in regulating cell cycle progression (Richardson *et al.*, 1992; Pines, 1993; Sherr,

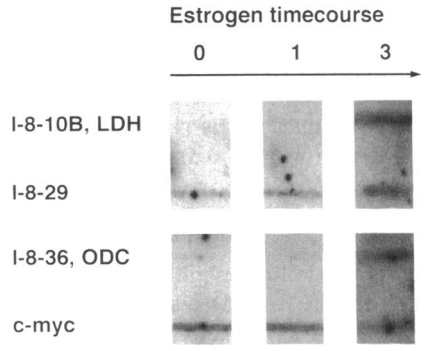

Figure 8. Nuclear run-on assay of myc regulated transcripts. MER #6 cells were seeded and brought to growth arrest by contact inhibition in medium containing 5% calf serum as indicated in MATERIALS AND METHODS. Cells were treated for 1 and 3 h with 100 nm β-estradiol and nuclei harvested as described in MATERIALS AND METHODS. Run-off transcripts were generated from freshly isolated nuclei, and approximately equal counts were added to each hybridization. Abbreviations: LDH, lactate dehydrogenase; ODC, ornithine decarboxylase.

1993). In both studies, cyclin-E RNA levels were prominently upregulated by conditional c-myc activity, and we found that the regulation is transcriptional. Because ectopic cyclin E expression is sufficient to drive cell cycle progression in human fibroblasts (Ohtsubo and Roberts, 1993), this raises the possibility that cyclin E is a direct myc target that is sufficient to drive the G_0 to S transition, although it does not rule out significant contributions to execution from other genes.

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